

## Clinic–Epidemiologic Study of Human Infection by Granada Virus, a New Phlebovirus within the Sandfly Fever Naples Serocomplex

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**Abstract.** Granada virus (GRV), a new phlebovirus within the Naples serocomplex, has been recently described in phlebotomine sandflies from Spain. The presence of anti-GRV immunoglobulin G (IgG) antibodies was investigated by indirect fluorescence assay (IFA) and neutralization test (NT) in 920 serum samples from the Granada population. By IFA, an overall GRV seroprevalence of 15.8% ( $N = 145$ ) was observed, significantly increasing up to 65 years. NT was positive in 18% of anti-GRV IFA-positive samples. IgG antibodies against Toscana virus (TOSV), a hyperendemic phlebovirus within Granada province, were detected in 40% of anti-GRV-positive cases. Anti-GRV IgM antibodies were detected in 36 (6.6%) of 547 acute-phase serum samples from individuals with febrile illness, exanthema, and/or acute respiratory infection. All positives were anti-TOSV IgM-negative. GRV may infect humans, with most cases being asymptomatic. The codetection of anti-GRV and anti-TOSV IgG antibodies could be attributable to cross-reactivity or exposure to the same transmission vector.

### INTRODUCTION

In Spain, up to date, Toscana virus (TOSV) has been the only member of the sandfly fever Naples serocomplex associated with human infection.<sup>1,2</sup> TOSV causes neurological infections, mainly lymphocytic meningitis and occasionally, meningoencephalitis and encephalitis.<sup>1–4</sup> TOSV has been seldom associated with non-neurological processes.<sup>5,6</sup>

Besides TOSV, other phleboviruses are involved in human disease in Mediterranean countries (i.e., sandfly fever Naples virus [SFNV] and sandfly fever Sicilian virus [SFSV]). Contrarily, these viruses are not neurotropic, being associated with febrile syndromes (Papatasi fever or 3-day fever) and less frequently, exanthema and/or self-limited acute respiratory infection, which take place during the warm seasons of the year.<sup>7,8</sup> Neither SFNV nor SFSV has been detected in humans or vectors in Spain.

An epidemiologic study of phleboviruses vectors carried out in southern Spain in 2003–2004 revealed that, apart from TOSV, a new phlebovirus named Granada virus (GRV) was detected in 11 of 103 pools of sandflies. The presence of this virus in phlebotomines has been reported later in other areas of Spain (Balearic Islands and Catalonia).<sup>9</sup> Phylogenetic analysis of the complete genome has confirmed that GRV belongs to SFNV serocomplex and is closely related to Massilia virus, another new phlebovirus described in southern France.<sup>10</sup> GRV infection rate in phlebotomines is approximately 0.2%, much higher than the rate of 0.05% reported for TOSV.<sup>2,9</sup>

Whether GRV causes infections in humans is still unknown. This work focused on two main objectives. First, a seroprevalence study to detect GRV IgG antibodies was carried out in asymptomatic individuals from Granada province (south of Spain), where GRV was first detected and isolated. Second, we analyzed the role of GRV in different infectious diseases

of unknown etiology from patients attended in our area during the warm months of the year when the vector is circulating.

### MATERIALS AND METHODS

#### Prevalence study of anti-GRV immunoglobulin G antibodies.

A total of 920 serum samples was retrospectively selected for the seroprevalence study and stratified by the participants' age and sex. Participants were representative of the Granada population on the basis of data from the National and Regional Institutes of Statistics (<http://www.ine.es/>). Sera had been collected from September to December of 2003 and frozen at  $-40^{\circ}\text{C}$  until use.

Specific detection of anti-GRV immunoglobulin G (IgG) antibodies was accomplished by an indirect fluorescence assay (IFA). Briefly, GRV antigen was prepared from the strain recovered in Vero cells. Cell cultures containing infected and uninfected cells were harvested and fixed on 18-well slides. IFA was carried out by adding 10  $\mu\text{L}$  serum sample to each well and incubating at  $35^{\circ}\text{C}$  for 30 minutes in humidity chamber. After incubation, human-specific fluorescein isothiocyanate (FITC)-labeled antiglobulins (Fluoline H; bioMérieux, Marcy-l'Étoile, France) were added to the wells and incubated at  $35^{\circ}\text{C}$  for 30 minutes as described above. Observation of the specific fluorescent foci by  $40\times$  field examination was recorded. Slides of negative controls were obtained with uninfected Vero cells.

IFA assays were carried out with twofold dilutions (1:20–1:640) of the serum samples. A serum dilution was considered positive when fluorescent foci were observed in slides with infected Vero cells and not in slides with uninfected cells. All sera were also tested for the presence of anti-TOSV IgG antibodies by a commercial enzyme immunoassay (EIA) (Enzywell Toscana Virus IgG; DIESSE, Siena, Italy).

All IFA-positive sera for GRV IgG antibodies were subjected to cytopathic effect neutralization test (NT)<sup>11</sup> carried out in Vero cell monolayers propagated in 96-well flat-bottom microtiter plates. A 100- $\mu\text{L}$  aliquot of twofold dilutions (1:10–1:1280) of each positive sample was mixed in parallel with 100  $\mu\text{L}$  of 100 TCID<sub>50</sub> (50% tissue culture

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infectious dose) of GRV strain and a TOSV strain, which is the other phlebovirus isolated from human disease in this area to date. Plates were incubated at 35°C and examined daily for the appearance of cytopathic effect in control wells inoculated with viral strains. A sample was considered positive when cytopathic effect was absent at least in the wells with the first two dilutions (virus neutralization titer  $\geq 20$ ).

**Clinical study to evaluate the role of GRV in human infection.** Acute-phase serum samples from 547 individuals, collected at days 5–10 after the appearance of symptoms, were used for the clinical study. The study was carried out between May and October of four consecutive seasons (2008–2011). The study population included persons from our healthcare area, with ages ranging from 1 to 87 years, from whom serum samples had been submitted to the laboratory for the investigation of common pathogens associated with the following clinical diagnosis: acute febrile illness (FI) lasting less than 7 days ( $N = 360$ ), exanthema ( $N = 33$ ), and/or acute respiratory infection (ARI;  $N = 154$ ). Routine serologic testing for the investigation of infectious agents related with the mentioned clinical syndromes was negative in serum samples from the study population.

The presence of anti-GRV IgM antibodies was investigated in a 1:10 dilution of each serum sample by IFA using the method described above. Human-specific FITC-labeled anti- $\mu$  IgM antiglobulins (Fluoline M; bioMérieux, Marcy-l'Étoile, France) were used as antiglobulins, and previous rheumatoid factor adsorption was performed (RF Absorbent Kit; Siemens, Marburg, Germany).

All anti-GRV IgM-positive serum samples were also tested for the presence of anti-TOSV IgM antibodies by a commercial EIA (Enzywell Toscana Virus IgM; Dienes, Siena, Italy).

Approval to use human specimens included in this project was granted by the Ethics and Research Committee of University Hospital Virgen de las Nieves (Granada, Spain).

**Statistical analysis.** Data were statistically analyzed with the SPSS 15.0 software (SPSS, Chicago, IL). Along with descriptive statistics, bivariate analysis was performed to compare epidemiological data with laboratory results by  $\chi^2$  test, and subsequent analysis of corrected residues was performed to compare variables two by two from statistically significant analysis by the  $\chi^2$  test. A  $P$  value  $< 0.05$  was considered statistically significant.

## RESULTS

Anti-GRV seroprevalence as detected by IFA was 15.8% ( $N = 145$ ). Seroprevalence was significantly age-dependent and increased up to 65 years old ( $P = 0.035$ ), above which it decreased (Table 1). No statistical differences were observed

TABLE 1

Results of the seroprevalence study of anti-GRV antibodies by IFA in the population from Granada province

Age groups (years)	Anti-GRV antibodies		Total
	<i>n</i>	Percent	
$\leq 15$	11	9.0	122
16–40	70	15.9	441
41–65	45	19.7	229
$> 65$	19	14.8	128
Total	145	15.8	920

TABLE 2

Results of the neutralization test for detection of anti-GRV and anti-TOSV IgG antibodies in 145 IFA-positive serum samples for anti-GRV antibodies

Anti-GRV (IFA)	Anti-TOSV (EIA)	Neutralization test		<i>n</i>
		Anti-GRV	Anti-TOSV	
+	+	+	+	8
+	+	+	–	4
+	+	–	+	11
+	+	–	–	35
Total				58
+	–	+	+	3
+	–	+	–	11
+	–	–	+	4
+	–	–	–	69
Total				87

in the prevalence ratio by sex (16.1% in men versus 15.5% in women). Anti-TOSV antibodies were detected in 25.3% ( $N = 233$ ) of the study population. Both anti-GRV and anti-TOSV IgG antibodies were present in 40% ( $N = 58$ ) of cases.

NT against GRV and TOSV was carried out in 145 IFA-positive GRV cases, from which 58 cases were also positive to TOSV by enzyme-linked immunosorbent assay (ELISA). Neutralizing anti-GRV antibodies were detected in 17.9% ( $N = 26$ ), yielding a seroprevalence ratio of 2.8%. Neutralizing anti-TOSV antibodies were observed in 17.9% ( $N = 26$ ) of total cases and 32.7% of 58 ELISA-positive cases (Table 2). Neutralizing antibodies against both viruses were observed in 42.3% ( $N = 11$ ) of cases.

The clinical study revealed anti-GRV IgM antibodies in 36 of 547 cases (6.6%). Anti-GRV IgM positivity ratios showed no statistical differences among the different clinical syndromes studied ( $P = 0.461$ ) (Table 3). All these positive sera were anti-TOSV IgM-negative.

## DISCUSSION

The 15.8% seroprevalence detected by IFA and the observation of 2.8% GRV-neutralizing antibodies (18% of IFA-positives) obtained in this study confirm that GRV might infect humans, which was suggested in another previous preliminary study.<sup>9</sup>

Although neutralization tests are considered more specific than IFA, negative results by the former do not exclude infection. Thus, positive results by IFA should not be always considered false positives when neutralization assays are negative. In fact, 67% of ELISA-positive TOSV cases were negative by neutralization assay, despite the fact that ELISA may be more specific than IFA, because it only includes TOSV recombinant antigens. Moreover, neutralizing TOSV antibodies were detected in seven ELISA-negative cases.

TABLE 3

Anti-GRV IgM antibodies in patients from Granada province with mild syndromes of unknown etiology

	Anti-GRV IgM antibodies		
	<i>n</i>	Percent	Total
Febrile illness	27	7.5	360
Exanthema	2	6.1	33
Acute respiratory infection	7	4.5	154
Total	36	6.6	547

This finding could not be analyzed for GRV, because a neutralization test was only carried out in IFA-positive cases, but we could speculate that this feature could take place. This lack of agreement among serologic assays for phleboviruses has been reported before.<sup>12</sup> In addition, in 2008, we recovered a TOSV isolate from a cerebrospinal fluid (CSF) sample of a patient with meningitis, and the neutralization assay to detect anti-TOSV IgM antibodies was negative (personal communication).

Although cross-reactivity in serologic tests is feasible among phleboviruses, the detection of anti-GRV and anti-TOSV antibodies within the same individual could be attributable to concomitant or more probably, sequential infection by both phleboviruses throughout life because of the exposure to the transmission vector that both viruses share. Anti-TOSV IgG antibodies, as assessed by a commercial EIA, were detected in 40% of GRV-positive cases by IFA, and 25% of TOSV-positive cases were also positive to GRV antibodies. Similarly, an epidemiological relationship between TOSV and *Leishmania infantum* infections has been previously suggested because of the common exposure to the vector *Phlebotomus perniciosus*. In a study carried out by Bichaud and others,<sup>13</sup> 51% of individuals with anti-TOSV IgG antibodies were also positive to *Leishmania* IgG antibodies, and conversely, 48% of individuals with *Leishmania* antibodies were positive to anti-TOSV IgG antibodies.

GRV seroprevalence increased with age, which occurs with TOSV,<sup>2,14</sup> possibly related with the greater probability of exposure to the transmission vector throughout the life. Curiously, GRV seroprevalence increases with age among the first three age groups, and it maintains similar or slightly lower seroprevalence in persons > 65 years, whereas TOSV seroprevalence also follows an age-dependent increase but is maximum in this age group.<sup>2,14</sup> This difference between both viruses may be because of differences in the period that each virus is circulating in this area. TOSV has been involved in human disease in Spain since 1988,<sup>1</sup> and GRV could be recently introduced or its numbers increased in our area because of climatic and/or ecologic changes that are taking place since the mid-20th century. Persons older than 65 years were not exposed to GRV when they were young, and thus, the seroprevalence in this age group is similar to the one detected in younger individuals. These data give additional support to the hypothesis that different infection by both phleboviruses within the same individual may happen, because cross-reactivity should have increased with age in GRV cases over 65 years.

Clinical investigation was focused on determining the role of GRV in syndromes commonly caused by other phleboviruses such as SFNV<sup>7,15</sup> (i.e., mild and self-limited FI, exanthema and ARI), with which GRV is closely related, as shown by phylogenetic studies.<sup>9</sup> Neurological processes were not included, because no phlebovirus other than TOSV was detected in any of 457 CSF samples received in our laboratory during the last 4 years. In the regional reference laboratory for diagnosis of viral meningitis and encephalitis (Andalusia, Spain), phleboviruses' investigation was routinely carried out in CSF samples by using a generic reverse-transcription-nested polymerase chain reaction (PCR)<sup>16</sup> (data not shown).

The results obtained in the serosurvey study (15.8% of GRV seroprevalence and 2.8% of neutralizing antibodies in the study population) and the detection of anti-GRV IgM

antibodies in 6.6% of mild syndromes of unknown etiology, negative to TOSV and other common pathogens, suggest that GRV could be involved in human infection in our area, which could be similar to the clinical implication of SFNV in Italy. The demonstration of anti-GRV IgG seroconversion between acute- and convalescent-phase serum samples would have provided confirmation of these findings. Because the study population was outpatients attended for a mild syndrome in primary healthcare services, the recovery of a representative sample of convalescent-phase sera was very difficult, and this analysis could not be carried out. Thus, during summer seasons, GRV investigation could be conducted in certain mild syndromes, in which other common etiologic agents have been discarded. The availability of a diagnostic assay for GRV might be encouraging in reference laboratories from certain geographical areas. Serologic tests that determine anti-GRV IgM antibodies can be feasible diagnostic tools.

The results obtained in this study show that GRV may infect humans in our area and that most cases would probably be asymptomatic. Occasionally, mild GRV infections may occur, usually as self-limited febrile illnesses accompanied by other signs and symptoms. These infections most probably take place during the warm months of the year, which occurs with infections caused by other phleboviruses in Mediterranean countries. Whether GRV or related viruses could be circulating in other areas or countries and/or causing mild febrile diseases needs to be determined. Additional epidemiological and clinical studies will assess the distribution and/or role of GRV in human infection.

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